Recovery and survival of nontuberculous mycobacteria under various growth and decontamination conditions

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The survival of microorganisms of the ***Mycobacterium avium, M. intracellulare,*** and ***M. scrofulaceum*** (MAIS) complex was evaluated after various soil and water decontamination regimens. Survival was reduced by growing cells in natural waters compared with laboratory media and by inclusion of malachite green in media as an antifungal agent. Decontamination with benzalkonium chloride, while reducing survival significantly less than 1% NaOH, failed to eliminate many fungi. Recovery from soil was further reduced by transfer losses and by irreversible cell adsorption onto particulates.

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La survie des microorganismes du complexe de ***Mycobacterium*** (MAIS): ***M. avium, M. intracellulare, M. scrofulaceum,*** a été évaluée, suite à des régimes de décontamination d'eau et de sol variés. La survivance était réduite lorsque les cellules croissaient dans des eaux naturelles, comparativement aux milieux utilisés en laboratoire, et suite à l'ajout d'un agent antifongique dans les milieux, le vert de malachite. La décontamination au chlorure de benzalkonium, bien que réduisant la survivance de façon significativement moindre que le NaOH 1%, n'a pas favorisé l'élimination de plusieurs des champignons. Le recouvrement en provenance des sols a davantage été réduit par les pertes de transfert et par l'adsorption irréversible des cellules sur les particulats.

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Introduction

The increased incidence of human infection by nontuberculous mycobacteria of the Mycobacterium avium, M. intracellulare, and M. scrofulaceum (MAIS) group and the absence of any evidence for transmission from person to person have stimulated efforts to find environmental sources for these pathogens (Wolinsky 1979). Consequently, MAIS organisms and other pathogenic mycobacteria have been sought and isolated from water (Beerwerth 1973; Kazda 1973; Goslee and Wolinsky 1976; duMoulin and Stottmeier 1978; Falkinham et al. 1980) and from soil (Jones and Jenkins 1965; Wolinsky and Rynearson 1968; Paull 1973; Suwankrughasn and Leat 1977).

Because mycobacteria grow slowly, even on enriched media, decontamination steps (e.g., oxalic acid, NaOH, or benzalkonium chloride) must be used for environmental samples to eliminate faster growing microorganisms. Such treatments usually elirninate the contaminants, but they also kill a significant proportion of the mvcobacteria. The onlv extensive studv of mycobacterial sensitivity to decontamination (Krasnow and Wayne 1966) concerned tubercle bacilli in sputum samples, and Songer (1981) has stressed the need for more information.

Because identification and evaluation of environmental reservoirs of MAIS organisms requires accurate counts of the organisms present and thus of the proportion recovered after decontamination, our objectives in this study were (i) to determine the recovery and survival of MAIS isolates from water and soil after a variety of published decontamination treatments (Jones and Jenkins 1965; Wolinsky and Rynearson 1968; Paull 1973; Falkinham et al. 1980), as well as after a treatment we developed for convenient, rapid decontamination of large numbers of soil samples; (ii) to examine with specific media, the influence of initial growth conditions on survival after various decontamination treatments (Krasnow and Wayne 1966; duMoulin and Stottmeier 1978; Merkal et al. 1981); and (iii) to examine the tolerance of MAIS strains for the fungal inhibitor malachite green.

Materials and methods

Bacterial isolates

The mycobacterial isolates used in this study, with their biovars and sources, are listed in Table 1. The biovars represent the four major classes of MAIS organisms isolated

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from water and air (Falkinham *et al*. 1980; Wendt *et al*. 1980).

Media and growth

Cells were grown either in autoclaved New River water (McCoy, VA) or in Middlebrook and Cohn 7H9 (M7H9) medium containing 0.5% (v/v) glycerol and 10% (v/v) OADC (oleic acid, albumin, dextrose, catalase) enrichment (BBL, Cockeysville, MD). Cultures or treated suspensions of MAIS organisms were plated for viable count determinations on Middlebrook and Cohn 7H10 agar medium containing 0.5% (v/v) glycerol and 10 or 4% (v/v) OADC enrichment ($M7H10^+$), both proving equally effective. Viable counts also were determined on M7H10 without enrichment ($M7H10^-$) or M7H10 containing 0.5% (v/v) glycerol, 10% (v/v) OADC, and 0.0025% malachite green ($M7H10^+$/Mal).

Comparison of cells grown in complex medium and in natural water

Isolates were grown in either M7H9 or autoclaved New River water to densities of 1×10^8 colony-forming units (cfu)/mL. A 1-mL portion of each culture was transferred into each of two sterile 10-mL centrifuge tubes and centrifuged at $8000 \times g$ for 20 min at 4°C. One pellet from each culture was resuspended in 10 mL of sterile 1% (w/v) NaOH, the other in 10 mL of sterile 2% (w/v) NaOH or sterile distilled water (control). After 30 min 0.05 mL of each tube was transferred into 5 mL of sterile 0.067 *M* KH_2PO_4 buffer at pH 6.5 (P buffer). Spread plates were inoculated with 0.1 mL of buffered cell suspension on $M7H10^-$ and incubated at 30°C in candle jars.

Comparison of two decontamination procedures

To compare the effects of our two previously used decontamination procedures (Falkinham *et al*. 1980) on the recovery and survival of MAIS organisms from natural water, test isolates were grown to early logarithmic phase in autoclaved New River water. Appropriate volumes of such water were inoculated with 5000 cfu of each isolate and decontaminated by the two methods described in Falkinham *et al*. (1980), except that for both methods the exposure to NaOH was for 30 min. The two decontamination methods differed by the suspension of cells in 2.0% (w/v) NaOH neutralized subsequently with an equivalence of 1 N HCl or suspension in 1.4% (w/v) NaOH neutralized subsequently with P buffer. Both methods were equally effective and produced similar survival and recovery of MAIS. Dilutions of cultures were also prepared to measure the efficiency of plating of the MAIS isolates on media containing malachite green.

Recovery and survival of MAIS from soil

Soil samples collected from Byrd Park, Richmond. VA, were dried overnight at 90°C, and ground with mortar and pestle to break up any clumps in which contaminating organisms might survive. Samples (5 g) of this soil were exposed to ethylene oxide (EtO) for 2 h at 38°C in slanted screw-capped test tubes (20 × 120 mm) (with loosened caps) in a sterilizer at Montgomery County Hospital, Blacksburg, VA. Samples (5 g) of the EtO-exposed soils were inoculated with ca. 5×10^5 cfu of each MAIS isolate and then exposed to either 4% NaOH – malachite green – cyclohexamide (Wolinsky and Rynearson 1968), 4% NaOH (Paull 1973), or 0.06 or 0.26% benzalkonium chloride (Jones and Jenkins 1965). All regimens were followed as described. except that colony counts were determined on $M7H10^+$/Mal for the procedures of Wolinsky and Rynearson (1968) and Paull (1973) and on $M7H10^+$/Mal containing 10% (v/v) egg yolk for the method of Jones and Jenkins (1965). Plates were incubated at 30°C in candle jars.

TABLE 1. Mycobacterial isolates used in this study

Origin and biovar"	Isolate	Source
Aerosal		
– – –	2s	James River, Richmond, VA
Water		
+++	JR151	James River. Richmond, VA
	W158	New York, Harbor, NY
	W200	Lake Borgne, New Orleans, LA
	W220	Mobile Bay, Daphne, AL
	JR1008	James River, Richmond, VA
+–+	JR124	James River, Richmond, VA
	W267	Claremont Beach. James River, VA
	W271	Port Mahon, Delaware Bay, DE
	W276	Stern's Pond, North Andover, MA
	JR995	James River, Richmond, VA
+– –	W279	Port Mahon, Delaware Bay, DE
	W478	Hudson River, NY
	JR1044	James River, Richmond, VA
	W1051	Fenholloway River, FL
– – –	W250	Stem's Pond, North Andover, MA
	W259	Port Mahon, Delaware Bay, DE
	JR1638	James River, Richmond, VA
Human		
– – –		
(*M. avium*)	Eissle	Chestertown, MD
	M16	NY
	788	NY
	8628	NY

"Biovar symbols designate the presence or absence of pigmentation. urease. and semiquantitative catalase activity, respectively.

Another set of inoculated soil samples was exposed to a simpler and faster procedure. Soil samples (5 g) were suspended in 20 mL of 0.1-strength nutrient broth (Difco, Detroit, MI) in sterile 50-mL polycarbonate centrifuge tubes. After manual shaking for 60 s, the suspensions were centrifuged at $1000 \times g$ for 10 min at room temperature to pellet the soil particles. A 10-mL portion of the supernatant was transferred to another sterile polypropylene centrifuge tube and centrifuged for 20 min at $8000 \times g$ at 4°C. The pellet was resuspended in 10 mL of 1% NaOH and immediately centrifuged for 10 min at $15\,000 \times g$ at 4°C. This pellet was

TABLE 2. Effect of initial growth conditions on survival after decontamination by 1 or 2% NaOH

Biovar or species	Isolate	Survival, %[a]			
		M7H9 grown		Water grown	
		1% NaOH	2% NaOH	1% NaOH	2% NaOH
+++	W158	15	ND[b]	2.0	0.6
	W200	42	ND	0.5	0.02
	W220	29	11	3.0	0.2
	JR1008	50	65	2.6	1.6
+−+	JR124	67	19	4.1	4.5
	W276	18	12	4.7	1.0
	JR995	57	ND	9.3	3.5
+−−	JR151	62	59	6.1	3.9
	JR1044	94	60	11.3	5.8
−−−	JR1638	41	51	5.2	4.9
M. avium (−−−)	Eissle	ND	ND	ND	5.1
	M16	27	12	4.8	2.5
	788	ND	ND	ND	0.8
	8628	ND	ND	ND	0.1
Mean[c]		47±24	36±24	4.9±3.1	2.5±2.1

[a]Average of duplicate determinations.
[b]ND, not determined.
[c]Mean ± standard deviation.

washed once in 10 mL of P buffer and centrifuged at 8000 × *g* for 20 min at 4°C. The final pellet was resuspended in the residual liquid (ca. 0.2 mL), and equal volumes were spread on M7H10 and M7H10$^+$/Mal.

Statistical analysis

The results obtained with various isolates, initial growth conditions, and decontamination methods were compared statistically by nonparametric tests and by the normal-distribution Student's t-test.

Results and discussion

Influence of initial growth conditions on survival

The survival of MAIS isolates grown in complex medium (M7H9) was significantly higher ($P < 0.001$ with 21 degrees of freedom by analysis of variance for both 1 and 2% NaOH decontamination) than for cells grown in water (Table 2). The data represent averages of duplicate determinations. Our results and those of Krasnow and Wayne (1966), duMoulin and Stottmeier (1978), and Merkal *et al.* (1981) agree that initial growth conditions influence the ability of mycobacteria to survive decontamination treatments. Further, there was a significant difference in survival of water-grown cells to 1 and 2% NaOH ($P < 0.005$, two tailed with 10 degrees of freedom by Student's t-test). No such difference was noted for M7H9-grown cells ($P > 0.1$, two tailed with 7 degrees of freedom by Student's t-test). Though there appeared to be wide variation of MAIS strain sensitivity to NaOH, there were too few representatives of the different biovars to allow any conclusions to be derived.

TABLE 3. Influence of malachite green on efficiency of plating of MAIS organisms

Biovar	Isolate	Efficiency of plating[a]
+++	W158	0.71
	W200	0.75
	W220	0.85
+−+	W267	0.75
	W271	0.043
	W276	0.72
+−−	W279	0.043
	W478	0.27
	W1051	0.59
−−−	2s	0.70
	W250	0.48
	W259	0.36

[a]Calculated as (cfu per millilitre on M7H10$^+$/Mal medium)/(cfu per millilitre on M7H10$^+$ medium). Average of duplicate deierminations.

Other experiments have demonstrated that representative isolates of the photochromogenic mycobacteria M. *marinum* and *M. kansasii* are extremely sensitive to NaOH (fewer than 0.1% of cells survived 2% NaOH). This probably explains our inability to isolate Runyon group I mycobacteria from water or soil samples (Falkinham *et al.* 1980; R. W. Brooks, B. C. Parker, H. Gruft, and J. O. Falkinham III, to be published). However, the lack of M. *avium* isolates from environmental samples (Falkinham *et al.* 1980) cannot be so explained, because M. *avium* appears to be no more NaOH sensitive than other MAIS strains (Table 2). Either the numbers of M. *avium* are very low (as a unique group or as rare mutants of other MAIS strains) or this species does not occur in water and soil but has a different origin for human and animal infection.

Effect of malachite green on efficiency of plating

Malachite green was inhibitory to a variety of MAIS isolates, whose survival on M7H10$^+$/Mal medium ranged from 4.3 to 85% of their survival on malachite green free M7H10$^+$ medium (Table 3). No biovar tested had a unique susceptibility or tolerance to malachite green though two isolates (W271 and W279) were far more susceptible than the rest (Table 3). Although the dye strongly inhibited some MAIS strains (e.g., 2 of 12), the presence of fungi in environmental samples makes its use desirable for recovery of MAIS organisms from a substantial fraction of soil samples.

TABLE 4. Comparison of survival of selected MAIS isolates suspended in water and subjected to either of two decontamination procedures

		Survival, %[a]			
		1.4% NaOH – P buffer		2.0% NaOH – 1 N HCl	
Biovar	Isolate	M7H10$^+$	M7H10$^+$/Mal	M7H10$^+$	M7H10$^+$/Mal
+++	W158	1.3	0.92	2.8	2.0
	W200	0.4	0.3	1.5	1.1
	W220	0.5	0.48	1.2	1.2
+−+	W267	2.3	1.7	5.2	3.9
	W271	1.2	0.05	0.6	0.03
	W276	0.4	0.29	0.4	0.29
+−−	W279	1.4	0.06	1.5	0.06
	W478	1.3	0.35	0.9	0.24
−−−	2s	1.9	1.3	3.3	2.3
	W250	0.9	0.43	0.4	0.19
	W259	0.2	0.07	0.9	0.32
Mean[b]		1.1±0.6	0.54±0.51	1.7±1.4	1.06±1.2

NOTE: Autoclaved water samples were each inoculated with 5000 cfu and treated with either 1.4 or 2.0% (w/v) NaOH. The NaOH was then neutralized with either P buffer or 1 N HCl, respectively.
[a]Average of duplicate determinations.
[b]Mean ± standard deviation.

Comparison of decontamination procedures

Recovery and survival from water

Not all cells suspended in natural water were recovered; a substantial proportion, which were not included in measuring survival, were lost during the centrifugation and transfer steps. Only an average of 27% of cells of a variety of strains originally suspended in water were recovered before the various decontamination regimens (data not shown). The data on survival from decontamination (Table 4) were not corrected for manipulation losses.

Of the MAIS organisms inoculated into natural water, there was little apparent difference in survival and recovery, regardless of which treatment was used (Table 4). However, it should be noted that two variables were influencing survival in this experiment; NaOH concentration (1.4 versus 2.0%) and neutralization (P buffer versus HCl). Because significantly more water-grown cells survived 1% compared with 2% NaOH (Table 2), the demonstration of higher recovery of cells after 2% NaOH decontamination followed by neutralization and resuspension in HCl (Table 4) suggests that P buffer was less effective in neutralizing alkalinity. That difference was only statistically significant ($P < 0.05$, two tailed with 10 degrees of freedom by Student's t-test) when cells were enumerated on medium containing malachite green. Though the inhibitory effect of malachite green was almost identical ($P = 0.995$ with 32 degrees of freedom by analysis of variance) with (Table 4) and without (Table 2) NaOH decontamination (note isolates W271 and W279), the difference in survival between the two regimens when cells were plated on medium containing the dye suggests that the combination exerts some additional influence on cell survival (i.e., they are not acting independently). As before, because too few representatives of each MAIS biovar were tested, no conclusions conceming their possible unique susceptibility can be drawn.

Recovery and survival from soil

In a cornparison of five procedures, only an average of 3.5% of the MAIS organisms inoculated into a soil sample were extracted before decontamination (Table 5). The remaining cells were irreversibly bound to the soils when the published procedures were employed (detergent extraction was not tested). The differences in extraction were significant at the $P < 0.005$ level (with 19 degrees of freedom by analysis of variance). These differences were due solely to the higher extraction by the Jones and Jenkins (1965) method, because there was no significant difference in extraction by the other three methods ($P \geq 0.5$ with 11 degrees of freedom by analysis of variance). The Jones and Jenkins (1965) method involves shaking the samples in distilled water and letting them stand overnight until the suspension clears. By contrast, in the Paull (1973) procedure the samples are shaken briefly in distilled water, and in both the Wolinsky and Rynearson (1968) and Brooks methods the samples are suspended and shaken briefly in a bacteriologic (complex) medium.

TABLE 5. Extraction and survival of MAIS organisms from soil after five procedures

Method (active decontaminant)	Effective concn.	Isolate[a]	% extracted[b]	Survivors[c] after decontamination as a percent of: no. extracted	inoculum
Jones and Jenkins (0.26% benzalkonium chloride)	0.13%	W220	5.2	100	5.2
		W1051	6.5	79	5.1
		W267	7.3	100	7.3
		2S	5.8	14	0.8
		Mean[d]	6.2±0.9	73	4.6
(0.06% benzalkonium chloride)	0.03%	W220	2.4	100	2.4
		W1051	7.1	79	5.6
		W267	8.1	100	8.1
		2S	5.6	15	0.8
		Mean[d]	5.8±1.5	73	4.2
Wolinsky and Rynearson (4% NaOH – malachite green – cyclohexamide)	2%	W220	1.3	5.1	0.07
		W1051	2.2	3.2	0.07
		W267	5.2	0.9	0.05
		2S	0.5	0.5	0.03
		Mean[d]	2.3±2.1	2.4	0.06
Paull (4% NaOH)	2%	W1051	0.8	2.8	0.02
		W267	1.1	3.1	0.03
		2S	0.3	0.2	0.001
		Mean[d]	1.2±0.9	1.9	0.02
Brooks (1% NaOH)	1%	W220	1.4	6.3	0.09
		W1051	1.3	5.0	0.07
		W267	5.1	4.1	0.21
		2S	0.3	0.3	0.001
		Mean[d]	2.0±2.1	3.9	0.09

[a]The isolates were selected to represent four biovars: W1051, +−−; W267, +−+; W220, +++; and 2S, −−−.
[b]Inoculated soil was suspended in the solution appropriate for the method employed. Viable cells in the supernatant were counted. Average of duplicate determinations.
[c]Average of duplicate determinations.
[d]Mean ± standard deviation.

Those differences in extraction efficiency suggest two possible explanations. First, the prolonged incubation of MAIS cells in distilled water containing soluble soil components could lead to their growth (George *et al.* 1980). Second, MAIS adsorption to soil could be mainly hydrophobic, unlike that of either *Escherichia coli* (Roper and Marshall 1974) or poliovirus (Taylor *et al.* 1981), whose adsorption is electrostatic. Since prolonged washing of soils to which MAIS cells have been added does not result in recovery of more than 20% of cells (G. Pabst and H. Gruft, unpublished data), hydrophobic interactions involving the lipid-rich mycobacterial surface could be major determinants of MAIS binding to soils. If that were to prove to be the case, inclusion of detergent (e.g., Tween 80) in aqueous extraction solutions might be useful.

The combination of benzalkonium chloride with medium containing egg yolk produced the greatest recovery of MAIS organisms after decontamination ($P = 0.001$ with 19 degrees of freedom by analysis of variance). However, the higher cost of this medium and the greater frequency of contamination by fungi makes the benzalkonium chloride procedure less desirable. Similarly oxalic acid (Beerwerth 1973) is an effective decontaminant, resulting in fungal overgrowth of most plates. In one study, the use of 2.5% oxalic acid resulted in 23.8% MAIS and 1.8% nonmycobacterial survival (data not shown).

Differences in the effective concentrations of NaOH made little significant difference ($P = 0.39$ with

11 degrees of freedom by analysis of variance) in MAIS survival (Table 5). One isolate (2S, biovar ---) was uniformly more susceptible to all five decontamination regimens partly owing to its lower rate of extraction from soil (Table 5). Because it was not uniquely susceptible to NaOH (Table 4), perhaps exposure to soil also induces some damage. In the two methods using only NaOH, survival was approximately two times higher at 1% (Brooks method) than at the 2% (Paull 1973) effective concentration though the differences were not significant ($P = 0.20$ with 7 degrees of freedom by analysis of variance). However, even 1% NaOH produced very low MAIS survival, and perhaps even lower concentrations would allow more MAIS survival while maintaining significant killing of other microorganisms. Owing to the small number of strains tested, statistical analysis of any strain differences was not attempted.

Our results confirm that recovery of MAIS organisms from environmental samples is a function of the independent processes of growth conditions, extraction, decontamination, and recovery medium composition. Extraction from water samples yields those organisms not adsorbed to the walls of centrifuge tubes and pipets and lost during transfer. The approximately 12-fold greater survival of the same MAIS isolates in water (Table 4, 2% NaOH – HCl on M7H10$^+$/Mal) relative to soil (Table 5, Brooks method mean) was due solely to the greater frequency of extraction from water before decontamination. EtO-treated (partially sterile) soils were preferred to autoclaved soils for our studies, since possible thermal modification of the organic fraction of soil, which contains most of the electrostatic binding sites (Alexander 1977; Brady 1974), might substantially influence MAIS absorption.

Our data suggest certain loss factors which, when multiplied by the number of cells recovered by a given method, should yield a reasonable estimate of the number in the original sample: for NaOH followed by plating on 0.0025% malachite green, a factor of 94 for water (2% NaOH) or 1100 for soil (1% NaOH); for 0.06% benzalkonium chloride and egg yolk medium a factor of 23.8 for soil. Studies using NaOH with natural, uninoculated soil samples indicated that the inocula used in these experiments represent the true cell densities in soils in the southeastern United States (R. W. Brooks, B. C. Parker, H. Gruft, and J. O. Falkinham III, to be published). These factors should serve until a more effective method which combines maximal extraction and minimal mortality from decontamination is developed.

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